

Chapter 10 – Biochemical Analysis of Gram Negative Species

Introduction and Background

Microbiologists will use a variety of agars to either identify the microbe using their respected biochemical properties that it displays once on that agar or to cultivate a specific microbe in an environment that it can only survive in. In this lab, we will be using two types of media to identify the biochemical properties of our microbes. These two types of media are selective media and differential media.

Selective media is intended to inhibit the growth of some microbes while permitting a select few to grow. Examples of selective media include, MacConkey agar and eosin-methylene blue agar (EMB agar).

Differential media will allow for the growth of more than one microbe of interest but will be able to differentiate species (colonies) by specific biochemical properties that are unique to the microbe. This will typically contain a substrate and/or a pH indicator. Examples of differential media include MacConkey agar, Eosin-methylene blue agar (EMB) and Triple Sugar Iron agar (TSI).

It is important to note that a particular media can be both selective and differential.

Below is description of the media that you will use in this lab:

MacConkey Agar:

This media is both selective and differential. This agar has crystal violet and bile salts, which will *inhibit* Gram Positive organisms and allow only gram-negative bacteria to survive. The Gram-Negative species that can tolerate bile are able to do this because their outer membrane is resistant bile.



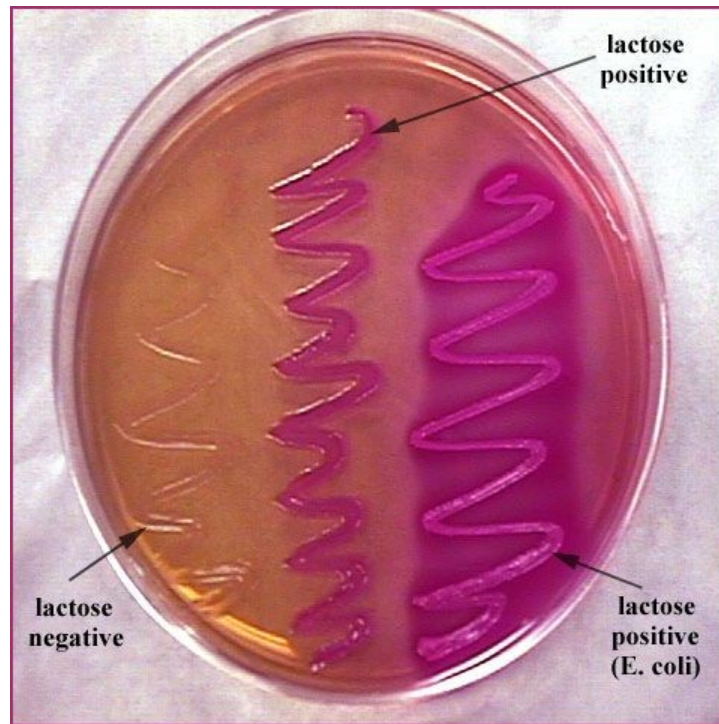
Picture #1: Example of uninoculated of MacConkey agar.

The agar also has the substrate lactose and the pH indicator neutral red. This will allow for the *differentiation* of Gram-Negative bacteria based if they have the enzymes present to *ferment lactose*.

When a microbe can ferment lactose it is using their enzymes and as result, they will produce an acidic waste product. This will lower the pH of the MacConkey agar and pH indicator neutral red will change the color of the agar pink. These microbes are considered *LACTOSE POSITIVE*.

When a microbe cannot utilize lactose because it may be lacking the enzyme to ferment lactose, the microbe will appear transparent. These microbes are considered *LACTOSE NEGATIVE*.

Please see picture #2. This is an example of how Gram-Negative microbes will appear if they are lactose positive or negative



Picture 2#: Inoculated MacConkey Agar.

Pre-lab question:

1. List the ingredients of MacConkey Agar.

Eosin Methylene Blue (EMB) Agar:

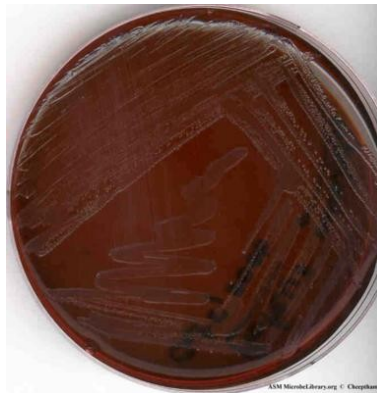
This agar is both selective and differential. This agar has the dyes eosin and methylene blue added, which *inhibit* gram-positive microbes from growing and will *select* for Gram-Negative organisms.



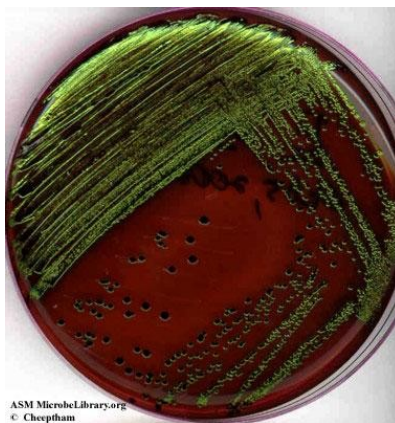
Picture 3: Uninoculated EMB agar.

EMB agar also contains lactose, which also allows for the *differentiation* between lactose positive and lactose negative organisms as spoke before in the MacConkey explanation.

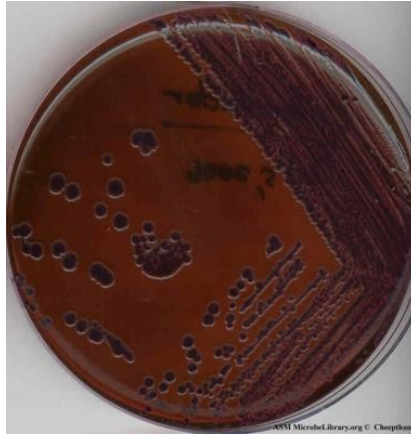
There are three possible colors that will be observed and each will differentiate the amount of acid production the microbe will make. Small amounts of acid production will result in a ***pink*** colored growth with black/purple centers in the center of the colony. Large amounts of acid production cause the acid to precipitate on the colony, which will result in a green, ***metallic sheen*** and with black/purple centers in the center of the colony. When there is no acid production, the colonies will be ***colorless***, therefore taking the color of the medium.



Picture #4: Lactose negative microbes on EMB agar.



Picture #5: Lactose positive with a large amount of acid production on EMB agar.



Picture #6: Lactose positive with a small amount of acid production on EMB agar.

Pre-lab questions:

2. What causes the green metallic sheen? Be specific.
3. List the ingredients for EMB agar.

Triple Sugar Iron Agar (TSI agar):

This agar is used to *differentiate* between various types gram-negative microbes. It is commonly called a TSI slant. This agar is in a test tube and is slanted. The slant is considered to be an aerobic environment while the bottom of the test tube (butt) is an anaerobic environment. TSI slants have 7 main ingredients.

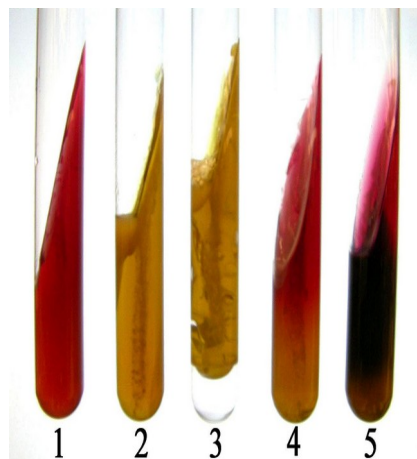
- o 0.1% glucose
- o 1.0% sucrose
- o 1.0% lactose
- o peptones
- o phenol red (pH indicator)

- o sodium thiosulfate
- o ferrous sulfate



Picture #7: Uninoculated TSI agar slant

Glucose, sucrose, and lactose are carbohydrate substrates for enzymes if present in bacteria. The bacteria will undergo fermentation of one, two or all three of the carbohydrates and create acidic end products. This will yield a yellow color on the slant and/or butt as the **acids** react with the phenol red. When the color yellow is observed, please indicate where it is and mention in your notes that the environment is acidic. This is also represented in test tube #2 in Picture #8.



Picture #8: Represents 5 different reactions in a TSI agar slant.

If the color red is observed that is an indication that the bacteria are utilizing the proteins in the agar as a substrate for their enzymes and as a result the bacteria are creating basic end products. This **alkaline** environment causes the indicator (phenol red) to turn red. This is best observed in test tube #1 in picture #8.

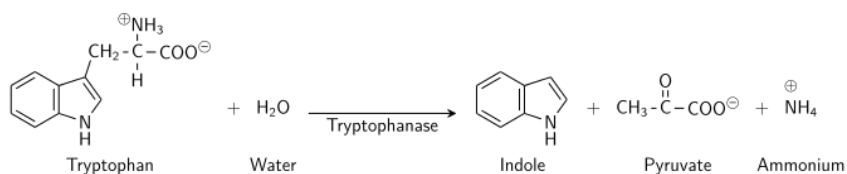
There are other instances that some bacteria may metabolize the sodium thiosulfate. In this instance, the bacteria will produce a

product of H_2S . This will react with the ferrous sulfate and yield a black color as observed in test tube #5 in picture #8.

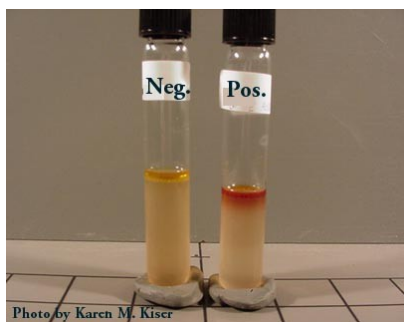
Finally, some bacteria may be able to produce a gas (H_2 or CO_2). This will be violent enough that it may push the butt of the agar off the bottom of the test tube or even rip the agar in half. This type of a reaction is observed in test tube #3 in picture #8.

Indole Test

This test will try and identify what species of bacteria have the enzymes to convert tryptophan to indole. The biochemical reaction is seen directly below.



Kovacs reagent is needed in order to observe this biochemical pathway since indole, pyruvate and ammonium are all colorless. When indole is metabolized by the microbe and Kovacs is added then you will have a **red** color appear on the surface of the liquid broth. This can be observed by picture #9 below. When the microbe cannot break down tryptophan to indole and Kovacs reagent is added then you will have a yellow color appear on the surface of the liquid broth.



Picture #9: Results of a indole test.

Materials

- o Wax Pencils (6 per Table)



- o Disinfectant Bottles (2 per table)



- o Test Tube Racks (2 per Table)



- o Metal Inoculating Loops (6 per Table)



- o Metal Inoculating Needles per table (6 per Table)



- o Bunsen Burners and Hoses (2 of each per table)



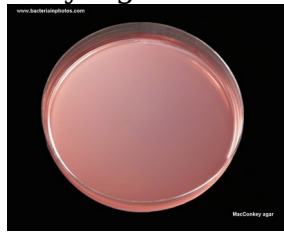
- o "Waste" 500ml Beakers (1 per table)



- o Gram Negative Agars/Slant Set-ups
 - o Each Set-up should have the following
 - o 4 EMB Agar Plates



- o 4 MacConkey Agar Plates



- o 9 TSI Slants



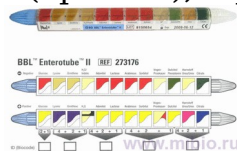
- o 9 Tryptone Broth Tubes



- o 1 Kovacs Reagent



- o 6 Enterotubes (optional), 1 per table



- o Metal Petri Dish Racks (1 per Table)



Microorganisms needed:

- o Citrobacter freundii*
- o Enterobacter aerogenes*
- o Escherichia coli*
- o Klebsiella pneumonia*
- o Proteus vulgaris*
- o Pseudomonas aeruginosa*
- o Salmonella enteritidis*
- o Serratia marcescens*

Procedures: Day 1

1. Obtain 9 TSI slant agar and label them with each organisms' name and one of the test tubes should be labeled "Negative Control". Do not write on the cap. Only write on the glass tube.
2. Obtain 9 Tryptone broths and label them with each organisms' name and one of the test tubes should be labeled "Negative Control". Do not write on the cap. Only write on the glass tube.
3. Obtain 5 EMB agar plates. With a marker, please dissect (label) one EMB at the plastic agar side of the plate in halves, two EMB plates should be dissected (labeled) in thirds on the agar and one EMB plate should be labeled negative control. The dissected portions of your EMB plates should be labeled with a different organism that you were provided with.
4. Obtain 5 MacConkey agar plates. With a marker, please dissect (label) one MacConkey at the plastic agar side of the plate in halves, two MacConkey plates should be dissected (labeled) in thirds on the agar and one MacConkey plate should be labeled negative control. The dissected portions of your MacConkey plates should be labeled with a different organism that you were provided with.
5. Inoculate the EMB and the MacConkey plates with their respected organisms aseptically. Do not inoculated the Negative Control Plate.

Caution: You do not need a lot of microbes! Gently spread them in their dissected portions. DO NOT overlap one microbe with an other!

6. Inoculate the TSI slant with their appropriate organism aseptically using the stab and streak method. **Do not stab the slant all the way down.** This will dislodge the agar the test and change your results. Do not inoculate the Negative Control.
7. Inoculate the Tryptone broths with their appropriate organism aseptically. Do not inoculate the Negative Control.
8. Incubate your plates, slants and broths at 35°C for 48 hours. Record the temperature of the incubator.
9. Return microorganisms back to the instructor's bench and disinfect your bench.

Optional:

10. Inoculate an Enterotube with a microorganism assigned to you by your instructor and incubate at 35°C for 48 hours.

Day 2

1. Take your plates, slants and broths out of the incubator. Record the temperature of the incubator.
2. Record your results of the EMB, MacConkey and TSI agars.
3. Take your Tryptone broths and add 6-7 drops of Kovacs reagent to each test tube including the negative control. DO NOT MIX THE TUBES. Record the results.
4. Tape your agar plates of EMB and MacConkey and dispose of them in the "Biohazard Waste"
5. Return all test tubes back to the instructor's bench and disinfect your bench.

Results:

MacConkey agar

Organisms	Color	Lac +/- Lac -	Other observations
<i>Citrobacter freundii</i>			
<i>Escherichia coli</i>			
<i>Klebsiella pneumonia</i>			
<i>Proteus vulgaris</i>			
<i>Pseudomonas aeruginosa</i>			
<i>Salmonella enteritidis</i>			
<i>Serratia marcescens</i>			
<i>Enterobacter aerogenes</i>			

EMB Agar

Organisms	Color	Lac +++ / Lac +	Other
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		/Lac -	observations
<i>Citrobacter freundii</i>			
<i>Escherichia coli</i>			
<i>Klebsiella pneumonia</i>			
<i>Proteus vulgaris</i>			
<i>Pseudomonas aeruginosa</i>			
<i>Salmonella enteritidis</i>			
<i>Serratia marcescens</i>			
<i>Enterobacter aerogenes</i>			

Indole test

Organisms	Color	Positive/Negative	Other observations
<i>Citrobacter freundii</i>			
<i>Escherichia coli</i>			
<i>Klebsiella pneumonia</i>			
<i>Proteus vulgaris</i>			
<i>Pseudomonas aeruginosa</i>			
<i>Salmonella enteritidis</i>			
<i>Serratia marcescens</i>			
<i>Enterobacter aerogenes</i>			

TSI agar

Organisms	Color of Slant, Acid/Alkaline	Color of Butt, Acid/Alkaline	H ₂ S Production, Color	Gas Production

<i>Citrobacter freundii</i>				
<i>Escherichia coli</i>				
<i>Klebsiella pneumonia</i>				
<i>Proteus vulgaris</i>				
<i>Pseudomonas aeruginosa</i>				
<i>Salmonella enteritidis</i>				
<i>Serratia marcescens</i>				
<i>Enterobacter aerogenes</i>				

Post Lab Questions:

1. Did *Proteus vulgaris* utilize a sugar in the TSI agar based off all your results including the EMB agar. If so, which one?
2. What type of conclusions can you make of *Pseudomonas aeruginosa* with respect to its ability to metabolize sugars?
3. How can you differentiate between *Escherichia coli* and *Citrobacter freundii*?